BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Attachment A

Summary

We have investigated a potential role for GacA, the response regulator of the GacA/GacS two component regulatory system, in *P. aeruginosa* biofilm formation. When *gacA* was disrupted in strain PA14, a 10 - fold reduction in biofilm formation capacity resulted relative to wild type PA14. However, no significant difference in the planktonic growth rate of PA14 *gacA*$^-$ was observed. Providing *gacA in trans* on the multi-copy vector pUCP-*gacA* abrogated the biofilm formation defect. Scanning electron microscopy of biofilms formed by PA14 *gacA*$^-$ revealed diffuse clusters of cells which failed to aggregate into microcolonies, implying a deficit in biofilm development or surface translocation. Motility assays revealed no decrease in PA14 *gacA*$^-$ twitching or swimming abilities indicating the defect in biofilm formation is independent of flagellar mediated attachment and solid surface translocation by pili. Autoinducer and alginate bioassays were similarly performed and no difference in production levels was observed, indicating this is not merely an upstream effect on either quorum sensing or alginate production. Antibiotic susceptibility profiling demonstrated that PA14 *gacA*$^-$ biofilms have moderately decreased resistance to a range of antibiotics relative to PA14 wild type. This study establishes GacA as a new and independent regulatory element in *P. aeruginosa* biofilm formation.

Introduction

Biofilms are adherent microcolonies of bacteria in which groups of cells are embedded within a complex and highly heterogeneous extracellular polymeric matrix (Costerton *et al.*, 1995). These consortia represent a unique mode of bacterial growth that is fundamentally different from planktonic or free-swimming cells. Infections resulting from pathogenic biofilms are characterised by a chronic or recurrent nature, and are highly resistant to conventional treatments (Costerton *et al.*, 1999). The basis for the persistent nature of biofilm infections is multi-factorial. One factor is that the biofilm mode of growth affords a certain degree of resistance to the host immune response (Jensen *et al.*, 1990; 1992; 1993, Anwar *et al.*, 1992; Meluleni *et al.*, 1995). A second factor is that biofilms have a two to three - order of magnitude decrease in susceptibility to antimicrobials compared to planktonically grown bacteria (Costeron *et al.*, 1999; Ceri *et al.*, 1999).

Pseudomonas aeruginosa, the archetypical opportunistic pathogen, has become the organism of choice for studying the physiological and genetic basis of biofilm formation, and phenotype. The suitability of this organism as a model system for understanding biofilms is three fold; it has a propensity for developing a variety of biofilm infections ranging from cystic fibrosis chronic pulmonary infections (Lam *et al.*, 1980; Singh *et al.*, 2000) to implant device related infections (Costerton *et al.*, 1999). Secondly, the bacterium is easily manipulated in the laboratory owing to its rapid rate of growth, sparse nutritional requirements and ability to develop highly resistant biofilm structures. Finally, genetic analysis of *P. aeruginosa* has been simplified as a result of the availability of the genome sequence (Stover *et al.*, 2000).

Genetic screens based on impaired attachment have identified a number of factors required for initial biofilm formation including flagella, Clp intracellular protease and many genes of unknown function (O'Toole and Kolter, 1998a). Further studies have demonstrated that type IV pili mediated surface motility, known as twitching, is required for microcolony aggregation, a secondary step required in biofilm maturation (O'Toole and Kolter, 1998b; O'Toole *et al.*, 2000). Davies *et al.* (1998) have further demonstrated

that the *las* quorum sensing system, but not the *rhl* system, is required for development of a mature biofilm architecture and for biofilm biocide resistance. However, Singh *et al.* (2000) have shown that *rhl* quorum sensing signal is elevated in biofilms relative to planktonically grown cells.

Of particular interest to our laboratories are the global regulatory genes which could influence the shift to and the maintenance of biofilm growth. These regulators may serve as targets for future antimicrobial therapies. As such, regulators that play a role in the virulence of the microbe as well as in its ability to form biofilms are of particular interest. A number of these regulators have been identified. LasR, the transcriptional activator of the *las* quorum sensing system, plays a role in both biofilm formation (Davies *et al.*, 1998) and virulence (Rumbaugh *et al.*, 1999a; Rumbaugh *et al.*, 1999b; Tang *et al.*, 1996). Recently, polyphosphate kinase (Rashid *et al.*, 2000) and the *crc* global carbon metabolism regulator (O'Toole *et al.*, 2000) were added to this group of genes. Our goal was to determine if other loci might also fit into a group of genes that are regulators of both biofilm formation and virulence.

The GacA/S two component global regulatory system has been demonstrated to be an essential virulence factor for *P. aeruginosa* pathogenesis independently in animal, plant, nematode and insect models of infection (Rahme *et al.*, 1995; Rahme *et al.*, 1997; Tan *et al.*, 1999a; Tan *et al.*, 1999b; Mahajan-Miklos *et al.*, 1999; Jander *et al.*, 2000). This regulatory system is comprised of GacS, the histidine kinase sensor protein (Barta *et al.*, 1992), and the cognate response regulator GacA, which has significant homology to the FixJ family of regulatory proteins (Rich *et al.*, 1994). While the signal to which GacS responds remains unknown, GacA has been shown to exhibit post transcriptional regulatory control of genes within the Gac regulon (Blumer *et al.*, 1999). In *P. aeruginosa*, GacA has been demonstrated to positively regulate the production of several virulence factors, specifically; N-butryl-L-homoserine lactone, pyocyanin, cyanide and lipase (Reimmann *et al.*, 1997). However, studies in other microorganisms have implicated much broader ranging effects, including regulation of toxins (Barta *et al.*, 1992; Rich *et al.*, 1994; Kitten *et al.*, 1998), proteases (Liao *et al.*, 1994; Grewal *et al.*,

1995), type III secretion (Hirano *et al.*, 1999), alginate biosynthesis (Liao *et al.*, 1996; Castaneda *et al.*, 2000), secondary metabolites (Whistler *et al.*, 1998), siderophores (Liao *et al.*, 1996; Zhang and Normack, 1996), swarming (Kinscherf and Willis, 1999) and invasion (Johnston *et al.*, 1996). Despite the diversity of functions regulated by GacA/GacS, the unifying theme that can be observed is that most products are extracellular and tend to play a role in the modification of the surrounding environment.

Because of the broad range of activities controlled through the *gac* regulon and its involvement in extracellular product formation we explored the potential involvement of the GacA/GacS two component regulatory system in biofilm formation of *P. aeruginosa*. In this study we demonstrate that GacA plays a critical role in biofilm formation, independent of factors previously identified to function in biofilm development. Furthermore, biofilms formed by a *gacA*$^-$ deficient strain of *P. aeruginosa* PA14 displayed a reduction in resistance to several different classes of antimicrobial agents. Together this data establishes that the *gacA* regulon acts to mediate biofilm formation through a novel pathway. It is further exciting to identify a factor key for the pathogenesis of *P. aeruginosa* involved in biofilm formation as this may suggest biofilms play a role in several previously identified infection models.

Results

A *gacA* mutant strain of *P. aeruginosa* is impaired in biofilm formation

To determine whether *gacA* plays a role in biofilm formation we compared the biofilm formation ability of a *gacA* mutant strain of *P. aeruginosa,* PA14 *gacA*$^-$, to two control strains, PA14 wild type and PA14 *toxA*$^-$. The PA14 *toxA*$^-$ strain was used as an additional control in these studies as it was engineered using the same Gm^R cassette used for the construction of PA14 *gacA*$^-$. Each population was grown in TSB in a MBEC^{TM} device with sampling over a 24 hour growth period. Biofilm samples were obtained from the pegs of the MBEC^{TM} device, and planktonic samples were taken directly from the growth vessel. Examination of growth rates over a 24 hour time period, revealed *P. aeruginosa* PA14 *gacA*$^-$ was defective in biofilm formation, as it formed biofilms at a reduced rate and with a 10 - fold reduction in final cell number (Figure 1A). This was not due to a defect in growth, as planktonic populations of *P. aeruginosa* PA14 *gacA*$^-$ proliferated at the same rates as PA14 wild type and PA14 *toxA*$^-$ (Figure 1B). Interestingly, there was no difference in biofilm formation or growth between PA14 wild type and PA14 *toxA*$^-$. This suggested that *toxA* likely does not play a role in biofilm formation and that the genetic manipulations done on both PA14 *toxA*$^-$ and PA14 *gacA*$^-$ did not influence the ability of the strains to form biofilms.

To add further support to the role of *gacA* in biofilm formation, complementation studies were performed by transforming PA14 wild type, PA14 *toxA*$^-$ and PA14 *gacA*$^-$ strains with pUCP-*gacA*. Biofilm growth rates of each transformed strain are shown in Figure 2. Biofilm formation by PA14 *gacA*$^-$ was restored when complemented *in trans* with pUCP-*gacA*, however, over-expression of *gacA* did not increase the biofilm formation rates of PA14 wild type or PA14 *toxA*$^-$ (Figure 2). Over-expression of *gacA* also did not significantly effect planktonic growth in any of the strains (data not shown). When transformed with only the vector control pUC181.8, strain PA14 *gacA*$^-$ maintained its biofilm deficient phenotype (data not shown). Biofilm formation rates of strains PA14 wild type and PA14 *toxA*$^-$ were not effected by the presence of the control vector alone. These results suggest that *gacA* is required for optimal biofilm formation and may regulate genes involved in biofilm development.

A *gacA* mutant of *P. aeruginosa* fails to aggregate to form microcolonies and mature biofilm structures

To determine if the biofilm formation defect of *P. aeruginosa* PA14 *gacA*⁻ was accompanied by morphological changes to the structure of the biofilms it formed, we performed scanning electron microscopy on biofilms formed by *P. aeruginosa* PA14 wild type, PA14 *toxA*⁻ and PA14 *gacA*⁻. Biofilms of each strain were grown in the MBEC™ device for 24 hours. Interestingly, differences in the amount of growth on the pegs could be seen unaided with the wild type cells producing large visible biofilms compared to a significant lack of a cell mass with PA14 *gacA*⁻. Visualisation of the growth on the pegs by scanning electron microscopy (SEM) was used to assess if differences in biofilm architecture existed. Figure 3 (A-F) shows that clear differences occurred in the biofilms formed by the three strains. PA14 *gacA*⁻ cells did adhere to the surface of the peg, but failed to aggregate and form microcolonies, even after 24 hours of growth. We also observed that whereas PA14 wild type and PA14 *toxA*⁻ colonization of pegs was for the most part restricted to large mats of biofilms, PA14 *gacA*⁻ colonization was sparse but uniformly distributed on the pegs. Thus, microscopic examination of biofilms formed by *P. aeruginosa* PA14 *gacA*⁻ also suggested that *gacA* is required for biofilm development.

Characterization of the biofilm formation defect of *P. aeruginosa* PA14 *gacA*⁻

The *gacA/gacS* regulons of several *Pseudomonas spp.* have been demonstrated to influence a number of factors including toxin and protease production and secretion, quorum sensing, alginate biosynthesis and swarming (Barta *et al.*, 1992; Rich *et al.*, 1994; Kitten *et al.*, 1998; Liao *et al.*, 1994; Grewal *et al.*, 1995; Liao *et al.*, 1996; Castaneda *et al.*, 2000; Kinscherf and Willis, 1999). While it is unlikely proteases and toxins play a role in biofilm formation, there is evidence that extracellular polysaccharides, quorum sensing and surface - associated motility are required for biofilm formation and development. Thus, we wanted to determine if the *gacA*⁻ - mediated defect in biofilm formation acts through any of the factors previously identified as being involved in biofilm formation.

a). Effect of *gacA* on quorum sensing in *P. aeruginosa*

Previous reports have suggested that GacA acts to enhance the transcription of *lasR* and thus influences autoinducer production (Reimmann *et al.*, 1997). As such, disruption of *gacA* may result in decreased LasR production, and hence autoinducer production and therefore explain the biofilm formation defect of *P. aeruginosa* PA14 *gacA*⁻. To determine if autoinducer production is altered in a strain PA14 *gacA*⁻ we measured the levels of autoinducer produced by each strain. The reporter strain *E. coli* MG4 (pKDT17) was used to measure the amount of N-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL) in the supernatants of stationary phase cultures of PA14 wild type, PA14 *toxA*⁻ and PA14 *gacA*⁻ (Table 1). The level of 3-oxo-C12-HSL produced by PA14 *gacA*⁻ is only slightly, and not significantly, diminished relative to PA14 wild type or PA14 *toxA*⁻. Likewise, because GacA has been reported to positively regulate N-butryl-L-homoserine lactone (C4-HSL) production (Reimmann *et al.*, 1997), we used the reporter strain *E. coli* DH5α (pECP61.5) to monitor the production of C4-HSL in the PA14 strains (Table 1). The production of C4-HSL was also only slightly decreased in PA14 *gacA*⁻ relative to the two control strains. We have also measured the timing of autoinducer production in strains PA14 wild type and PA14 *gacA*⁻ and these strains do not dramatically differ in the timing of either C4-HSL or 3-oxo-C12 HSLs (Sandhu and Storey, Unpublished observation). These results suggest that the autoinducer production in strain PA14 is not dramatically influenced by a mutation in GacA.

Previous research has shown that if *lasR* is present on a multicopy plasmid (pMJG1.7), LasR and autoinducer production is increased (Kirkham and Storey, Unpublished data). Thus, to compensate for the disruption of a potential positive regulator of *lasR*, we transformed the *lasR* over-expression vector pMJG1.7 into PA14 wild type, PA14 *toxA*⁻ and PA14 *gacA*⁻. We then examined resulting biofilm and planktonic growth rates. The presence of multiple copies of *lasR* did not bypass the biofilm formation defect of PA14 *gacA*⁻ as *P. aeruginosa* PA14 *gacA*⁻ (pMJG1.7) produced biofilms at the same decreased rate (approximately 10 fold lower than PA14) as PA14 *gacA*⁻ (data not shown). Furthermore, over-expression of *lasR* in strains PA14 wild type (pMJG1.7) and PA14 *toxA*⁻ (pMJG1.7) did not increase biofilm formation ability of these strains. Taken

together these experiments suggest that in strain PA14 an alteration in quorum sensing could not explain the decrease in biofilm formation that we see with PA14 *gacA*⁻.

(b). Effect of *gacA* on motility of *P. aeruginosa*

O'Toole and Kolter (1998b) demonstrated that twitching motility was necessary for microcolony aggregation, an initial step in biofilm formation. The GacA/GacS two component regulatory system has been shown to regulate the solid surface translocation of *P. syringae* (Kinscherf and Willis, 1999). To assess if the PA14 *gacA*⁻ biofilm formation defect was mediated through a defect in solid surface translocation, twitching motility and swarming assays were performed on each PA14 strain. *P. aeruginosa* PA14 wild type, PA14 *toxA*⁻ and PA14 *gacA*⁻ were stab inoculated into thin agar rich media plates, and the zones of twitching monitored after 24 and 48 hours. Zones of twitching were identical for each strain tested, indicating that there is no defect in type IV pili-mediated twitching motility in *P. aeruginosa* PA14 *gacA*⁻ (Table 1). Swarm assays similarity did not show that PA14 *gacA*⁻ had any impairment in its ability to swarm (Table 1). Notably, the mutation in *gacA* seems to enhance the ability of the bacteria to swarm (Table 1). As such, the biofilm formation defect of strain PA14 *gacA*⁻ is likely not mediated by a decrease in solid surface translocation.

Flagellar function has similarity been shown to be necessary for initial bacterial attachment and subsequent biofilm formation (O'Toole and Kolter, 1998a). We therefore assessed flagellar function of each of the three strains using flagellar swim plates. No difference in flagellar function was observed among the strains indicating that the PA14 *gacA*⁻ biofilm formation defect is not mediated through this pathway (Table 1).

(c). Effect of *gacA* on alginate production in *P. aeruginosa*

Studies in other bacteria have demonstrated that alginate production is up regulated by the *gacA/gacS* regulon (Liao *et al.*, 1996; Castaneda *et al.*, 2000). To assess if the biofilm formation defect of PA14 *gacA*⁻ was due to altered alginate production alginate bioassays were performed on each of the strains. Table one shows that PA14 *gacA*⁻ had a slight, but not significant, increase in alginate production over the wild type strain.

Effect of *gacA* on biofilm antibiotic susceptibility of *P. aeruginosa* PA14 strains

The fundamental feature associated with biofilm growth is drastically increased resistance to antibacterial agents. As such, the disruption of a genetic factor required for biofilm formation may result in a corresponding decrease in resistance to an antibiotic challenge. To examine if the biofilm formation defect observed in PA14 *gacA*⁻ resulted in an altered antimicrobial resistance profile, MBEC™ antimicrobial susceptibility testing was performed (Ceri *et al.*, 1999). MIC and MBEC values were determined based on absorbance readings of the antibiotic challenge plate and the recovery plate respectively. Little difference in planktonic antibiotic susceptibility was observed between the genetic backgrounds. A moderate decrease in biofilm antibiotic resistance to azythromycin, chloramphenicol, erythromyin, piperacillin and tetracycline was observed for PA14 *gacA*⁻ relative to PA14 wild type and the PA14 *toxA*⁻ control (Table 2). In order to measure the number of viable biofilm cells remaining after antibiotic challenge at each concentration, a subsequent MBEC assay was performed on the two isogenic strains PA14 *toxA*⁻ and PA14 *gacA*⁻ strains in which biofilm cells released by sonication into the recovery plate were serial diluted and spot plated to determine remaining viable CFU/peg (Figure 4). The number of viable biofilm cells remaining after antibiotic exposure was significantly decreased in PA14 *gacA*⁻ relative to PA14 *toxA*⁻ for all antibiotics tested; azythromycin, chloramphenicol, erythromycin, polymyxin B, and tobramycin. We postulate that the observed decrease in biofilm antibiotic resistance of strain PA14 *gacA*⁻ is the result of an inherent biofilm formation defect of this strain, resulting from the disruption of a key regulatory element required for biofilm formation and antibiotic resistance.

Discussion

The virulence of *P. aeruginosa* is multifaceted. Numerous virulence determinants are involved and we are just beginning to realise the importance the biofilm mode of growth plays in infections (Costerton *et al.*, 1999). Thus, an understanding of genes that are involved in both virulence and the biofilm mode of growth may lead to new classes of antibacterial agents with efficacy against biofilms at physiologically achievable levels. GacA, part of the GacA/S two component global regulatory system, has been shown to be

involved in the virulence of *P. aeruginosa* in a wide range of organisms (Rahme *et al.*, 2000) but its role in biofilm formation was previously unrecognized. In this study we identified a role for GacA in biofilm formation, and have further proceeded to examine the nature of this system in *P. aeruginosa* biofilm formation.

Reduced biofilm formation has been shown to result following the disruption of a number of genes involved in surface attachment and in the early stages of biofilm formation (O'Toole and Kolter 1998a; Davies *et al.*, 1998). Mutants with disruptions in key regulatory genes also seem to have reduced ability to form biofilms (Davies *et al.*, 1998; O'Toole *et al.*, 2000). Similarly, we showed that a *gacA* mutant has a ten-fold drop in the ability to form biofilms (Figures 1A). This was not a general growth defect as PA14 *gacA*$^-$ grows as well as wild type cells during planktonic growth (Figure 1B). Furthermore direct observation and comparison of PA14 *gacA*$^-$ to PA14 wild type revealed that the *gacA* mutant attached to surfaces but did not progress beyond the accumulation of a few cells (Figure 3). In contrast, PA14 wild type and *toxA*$^-$ formed dense multi-layered biofilms. Taken together these observations indicate that GacA plays an important role in biofilm formation.

Because the *gacA/gacS* regulon in other Pseudomonads has been demonstrated to effect solid surface translocation, alginate biosynthesis and autoinducer production (Chancey *et al.*, 1999; Kinscherf and Willis, 1999; Kitten *et al.*, 1998), and these functions have also been shown to be involved in *P. aeruginosa* biofilm formation (Davies *et al.*, 1998; Boyd and Chakrabarty, 1995; O'Toole and Kolter 1998b), we wished to assess whether the *gacA* defect in biofilm formation was mediated through any of these previously identified mechanisms. To examine the role of the GacA regulator on the regulation of the *P. aeruginosa* quorum sensing systems, we examined autoinducer production by the three *P. aeruginosa* PA14 strains, as well as examined the effects of over-expressing LasR on the biofilm formation defect of *P. aeruginosa* PA14 *gacA*$^-$. Assays of 3-oxo-C12-HSL production showed no significant differences in either timing of production or production levels of 3-oxo-C12-HSL in PA14 *gacA*$^-$ relative to the control strains (Table 1). Thus,

our results suggested that production of 3-oxo-C12-HSL autoinducer was not dramatically altered in PA14 *gacA*⁻ growing planktonically.

In *P. aeruginosa* the LasR-LasI-3-oxo-C12-HSL quorum sensing system is intertwined with the RhlR-RhlI-C4-HSL quorum sensing system. However, the role of C4-HSL in biofilms is as yet undefined. Whereas Davies *et al.* (1998) reported that C4-HSL is not required for biofilm formation nor biocide resistance, Singh *et al.* (2000) have reported elevated levels of C4-HSL as being a molecular marker of biofilm growth. Thus, we examined production of C4-HSL in *P. aeruginosa* PA14 *gacA*⁻ and the two control strains using a similar bioassay. C4-HSL production is slightly but not significantly decreased in PA14 *gacA*⁻ (Table 1) relative to that of strain PA14. Again, this suggests that RhlI and C4-HSL are again not altered in PA14 *gacA*⁻ as compared to the parental strain growing planktonically. A possible explanation for the differences in autoinducer production between PAO1 and PA14 could be due to altered regulation or perhaps a different complement of genes between the two strains.

Reimmann *et al* (1995) showed that in strain PAO1 GacA enhanced *lasR* production and so influenced autoinducer production. Research in our lab has shown that over-expression of the LasR in *P. aeruginosa* acts to markedly increase production of both 3-oxo-C12-HSL and C4-HSL (Kirkham and Storey, unpublished data). To overcome the potential deficit of LasR in strain PA14 *gacA*⁻ we over-expressed *lasR* on a multiple copy vector in PA14 *gacA*⁻. We then used this strain to determine if this could at least partially complement the biofilm formation ability of this strain. The over-expression of *lasR* from the vector pMJG1.7 in *P. aeruginosa* PA14 *gacA*⁻ did not bypass the biofilm formation defect of this strain (data not shown). Taken together these studies may suggest that the biofilm formation defect caused by a disruption of the GacA system is independent of the *las* and the *rhl* quorum sensing systems.

Twitching motility and swarming are types of solid surface translocation implicated in microcolony aggregation and subsequent biofilm formation (Semmler *et al.*, 1999; Pratt and Kolter, 1998; O'Toole and Kolter 1988b; O'Toole *et al.*, 2000; Kohler *et al*, 1999;

Rashid *et al.*, 2000). Twitching motility is believed to be mediated through the extension and contraction of type IV pili (Bradley, 1980; Semmler *et al.*, 1999). The electron micrographs in Figure 3 suggest that PA14 *gacA*⁻ lacks the ability to translocate across the surface of the pegs and so can't form microcolonies that would allow further maturation of the biofilm. These assays revealed identical zones of twitching for PA14 *gacA*⁻ and the two PA14 control strains (Table 1) indicating that twitching is not altered in a *gacA*⁻ mutant. Interestingly, in *P. aeruginosa* strain PA14 we show that a mutant in GacA has enhanced swarming ability (Table 1). This result could be explained in two ways. First, it is possible that the enhanced ability to swarm may be detrimental to biofilm development and this is the reason that a *gacA*⁻ mutant can't form a biofilm. The second possibility is that a GacA regulated mechanism of surface translocation other than twitching and swarming motility is needed for a biofilm to develop. We currently favour this second possibility.

Both flagella function and alginate production have been demonstrated to be important in biofilm formation and development (O'Toole and Kolter, 1998a; Boyd and Chakrabarty, 1995). We performed flagellar swim tests and alginate biosynthesis assays to assess if the *gacA* mediated defect was operating through either of these pathways. There was no difference in the ability of PA14 *gacA*⁻ to swim, relative to the two control strains (Table 1), indicating that the *gacA* mediated biofilm formation pathway is independent of flagellar motility. We also carried out alginate assays on PA14 and PA14 *gacA*⁻ and found only a slight increase in alginate production in the *gacA*- mutant (Table 1). However, given the variability of this assay on strains that produce relatively low amounts of alginate we do not think this difference is enough to account for alterations in the ability of the strains to form biofilms.

The fundamental feature associated with biofilm growth is their recalcitrant resistance thereby making them less susceptible to antimicrobial treatments than comparable planktonic bacteria (Costerton *et al.*, 1999). Thus, biofilm antibiotic susceptibility

profiling of *P. aeruginosa* PA14 *gacA*⁻ was performed to examine if the biofilm formation defect translated into a decrease in antimicrobial resistance. A two to four-fold decrease in minimal biofilm eradication concentration (MBEC) (Ceri et al. (1999)) was observed to the antibiotics azythromycin, chloramphenicol, erythromyin, piperacillin and tetracycline in PA14 *gacA*⁻ relative to PA14 wild type and the PA14 *toxA*⁻ control (Table 2). This increase in antibiotic sensitivity was not as profound during planktonic growth as evident by similar minimal inhibitory concentrations (MIC). For both the MBEC and the MIC assays we are measuring the concentration at which total killing takes place. Examination of cell viability, where we measure the antibiotic concentration that results in a three log reduction in cell numbers, proved even more interesting. *P. aeruginosa* PA14 *gacA*⁻ biofilms survived antibiotic challenge with far fewer viable cells than comparable *P. aeruginosa* PA14 *toxA*⁻ biofilms exposed to the same concentration of antibiotic (Figure 4). The number of viable biofilm cells remaining after antibiotic exposure was significantly decreased in PA14 *gacA*⁻ relative to PA14 *toxA*⁻ for all antibiotics tested; azythromycin, chloramphenicol, erythromycin, polymyxin B, and tobramycin. This trend was most pronounced with the antibiotics chloramphenicol and tobramycin. With these antibiotics there is a three to four-fold log reduction in biofilm survival following exposure to these antibiotics in *P. aeruginosa* PA14 *gacA*⁻ relative to PA14 *toxA*⁻ over all concentrations (data not shown).

The antibiotic resistance profile of *P. aeruginosa* PA14 *gacA*⁻ was somewhat surprising. Despite a 10-fold decrease in biofilm formation and final cell mass, and failure to mature into a dense bacterial biofilm, this knockout strain still demonstrated relatively high level of antibiotic resistance. SEM analysis revealed that little biofilm architecture was developed by this mutant strain, despite normal levels of alginate production. These data imply that the biofilm matrix, though it may serve as a diffusion barrier to antibiotics, does not account for the bulk of antibiotic resistance observed in biofilms. This is not surprising as previous studies have demonstrated that biofilm architecture acts to decrease the diffusion rate of several antibiotic classes, however, does not act to completely block penetration (Stewart, 1994; Nichols *et al.*, 1989; Suci *et al.*, 1994). It is

likely that the biofilm antibiotic resistant phenotype is cumulative and contributed by multiple factors, of which reduced permeability may only be one.

From these data we can conclude that GacA, a factor involved in multi-host virulence, plays a critical role in biofilm formation. Furthermore, the GacA regulatory system may regulate an alternate pathway required for optimal biofilm formation. In Strain PA14 this regulatory system seems to be independent of the *las* and *rhl* quorum sensing systems, alginate production and swimming, and twitching motility. Interestingly, swarming is enhanced in strain PA14 *gacA*$^-$ suggesting that in *P. aeruginosa* swarming is repressed by GacA. At present data is not available regarding the pathway through which the GacA/GacS two component regulatory system acts to mediate biofilm formation. It does appear that in the PA14 *gacA*$^-$ surface translocation is altered. Identification of the regulatory cascade through which *gacA* acts to effect biofilm formation potential is essential to understanding the molecular and genetic basis of biofilm development and maturation. Furthermore, identifying the signal to which GacS (LemA) responds to and initiates expression of the genes within the *gac* regulon is required in order to fully understand the role the GacA/GacS two component regulatory system plays in *P. aeruginosa* biofilm formation. The identification of *gacA/gacS* two component regulatory system involvement in biofilm formation and antibiotic resistance is important to both the understanding of biofilm development, and furthermore in establishing an *in vitro* role for factors critical *in vivo*.

Experimental Procedures

Bacterial strains and media

Bacterial strains and plasmids used in these studies are listed in Table 3. *P. aeruginosa* strain PA14 and its *toxA*⁻ and *gacA*⁻ derivatives, PA14 *toxA*⁻ and PA14 *gacA*⁻ respectively, were used in all biofilm formation studies (Rahme *et al.*, 1995). Unless otherwise indicated, strains were grown in tryptic soy broth (TSB) (BDH) at 35°C with 95% relative humidity. All enzymes used for DNA manipulations were purchased from Gibco BRL. All plasmid constructs were maintained in *E. coli* JM109 using standard protocols (Ausubel *et al.*, 1991), and then transformed into *P. aeruginosa* by electroporation (Smith and Iglewski, 1989). Antibiotics were added to the following concentrations: (i) *E. coli*, ampicillin, 100 µg/ml (ii) *P. aeruginosa* carbenicillin, 400 µg/ml.

Biofilm and planktonic growth curves

All growth curve manipulations were performed in BioSafety laminar flow cabinets to reduce the possibility of contamination. The MBEC™ device (MBEC Biofilm Technologies Limited) was used to form biofilm and planktonic populations (Ceri *et al.*, 1999). The inoculum was formed from an overnight culture grown on solid media (tryptic soy agar (TSA) or TSA supplemented with 400 µg/ml carbenicillin). The secondary culture used to inoculate the device consisted of 25 ml of a 1 X 10^7 CFU/ml dilution in TSB (supplemented with 400 µg/ml carbenicillin when necessary). The MBEC™ device was incubated at 35°C on a rocking table (Red Rocker set at speed 4.5, Hoefer Instrument Co.) to generate the shear force necessary for biofilm formation. Biofilm samples were obtained by removing individual pegs from the lid of the device using sterile pliers. Biofilm pegs were added to sterile 0.9% saline and then sonicated using an ultra-sonic cleaner (Aquasonic Model 250 HT; VWR Scientific) to disrupt the biofilm thereby releasing individual component cells. Planktonic populations were sampled by removing a defined volume of batch culture from the trough. To enumerate samples, serial dilutions and spot plating were performed. Each growth curve was performed in duplicate and the averages are shown.

Biofilm antibiotic susceptibility testing

The antibiotic susceptibility profiles of *P. aeruginosa* biofilm and planktonic populations were obtained following the methods of Ceri *et al.* (1999). The MBEC™ device (MBEC Biofilm Technologies Limited) was used to form 96 equivalent biofilms for biofilm antibiotic susceptibility profiling. Bacterial inoculums were formed as described above. Samples were grown until biofilms had developed to a population size of approximately 10^6 cells/peg (5-6 hours post inoculation) and then briefly rinsed to eliminate residual planktonic bacteria. The biofilm lid was then transferred to the 96 well microtitre antibiotic challenge plate.

Antibiotic challenge plates were constructed such that multiple antibiotics were tested simultaneously in each assay using 96 well microtitre plates. Antibiotics used in the challenge plate were serial diluted in cation adjusted-Mueller Hinton Broth (CA-MHB). Doubling dilutions were performed to generate a concentration gradient ranging from 1024 μg/ml to 2 μg/ml. Both a growth control lane and a sterility control lane were also used to confirm growth and absence of contamination. Biofilms were challenged for 16 – 20 hours at 35°C with constant shear force. After antibiotic challenge, the biofilm lid was briefly rinsed in microtitre plates and then transferred to a recovery mirotitre plate containing CA-MHB. Biofilms were disrupted to release individual component cells into the recovery media by sonication in an ultra-sonic cleaner for 5 minutes. Biofilm size was directly measured following antibiotic challenge. Each sample of the 96 well microtitre plate was serial diluted in 0.9 % saline to determine exact CFU remaining in the biofilm following antibiotic challenge. Alternatively, recovery plates were incubated overnight to allow for growth of any remaining bacteria. Minimal biofilm eradication concentrations (MBEC) are defined as the minimum concentration of antibiotic which prevents growth in the recovery plate. The antibiotic challenge plate was similarly read to determine presence or absence of growth. The minimal inhibitory concentration (MIC) refers to the minimum concentration of antibiotic which prevents planktonic growth.

Assays for autoinducer production

To determine if there was a difference in autoinducer production between *P. aeruginosa* PA14 wild type, PA14 *toxA*⁻ and PA14 *gacA*⁻, bioassays using *E. coli* reporter strains were performed. To accurately quantify the levels of autoinducer being produced in each strain examined, the liquid culture assay of Pearson *et al.* (1994) was employed. *P. aeruginosa* strains were grown in either LB broth or PTSB media and *E. coli* strains were grown overnight in A medium supplemented with 100 µg/ml of ampicillin.

To detect 3-oxo-C12-HSL, the A medium supplemented with *P. aeruginosa* supernatants, was inoculated with 3-oxo-C12-HSL reporter *E. coli* MG4 (pKDT17) (*lasB'-lacZ*) to an A_{540} of 0.1 and grown for 5-6 hours at which point the A_{600} was measured as an indication of growth. β-galactosidase procedures were then carried out as described by Miller (1972). To measure C4-HSL levels, the medium was inoculated with C4-HSL reporter *E. coli* DH5α (pECP61.5) (*rhlA'-lacZ*) to an A_{540} of 0.08 and grown at 37°C to an OD of 0.3. 1 mM IPTG was then added and the cells grown for an additional hour to further induce activity (Pearson *et al.*, 1997). β-galactosidase activity was measured as previously described (Millar (1972).

Twitching motility assays

To assess twitching motility of *P. aeruginosa* PA14 wild type, PA14 *toxA*⁻ and PA14 *gacA*⁻ zones of twitching were measured and compared. On very thin LB or TSA plates (< 2mm thick), each of the three PA14 derivative strains were inoculated using a stab loop. Bacterial proliferation between the agar and the surface of the plate was measured as the zone of twitching. Twitching zones were measured for each strain after 24 and 48 hours. To facilitate visualization of twitching zones, cells were stained with Coomassie Brilliant Blue G-250. After the defined incubation period, the agar was carefully removed and 8 ml of Coomassie Brilliant Blue G-250 was added and incubated for 2 minutes to stain the cells in the adherent zone of twitching. The surface of the petri dish was then rinsed twice with methanol to remove excess stain. Blue stained zones representing the zones of twitching were measured. Each assay was performed in triplicate and the average results are shown.

Flagellar swim plates and swarming assays

Flagellar swimming and swarming assays were performed as previously described by Kohler *et al.* (2000). Swim plates were incubated at room temperature and swarm plates were incubated at 37oC. All plates were grown for 72 hours. Each assay was performed in duplicate and the average results are shown.

Alginate biosynthesis assays

The alginate bioassay was performed using the modified carbazole assay described by May and Chakrabarty (1994). Each assay was performed in duplicate and the average results are shown.

Preparation of specimens for scanning electron microscopy (SEM)

Biofilm samples were fixed to MBEC™ device pegs for SEM as follows. 8 samples, representing pegs found in a single column of the MBEC™ device, of each strain were fixed during each procedure. Fixation of samples was performed using 96 well microtitre plates. 200 µl of each solution was added to each well in a column of the microtitre plate. The biofilm samples were fixed to the peg by incubation in a 5% glutaraldehyde/cacodylic buffer for 2 hours at room temperature. After fixation, samples were washed for 10 minutes in 0.1 M Cacodylic acid. This wash was repeated a total of five separate times. The samples were then washed in double distilled water to remove the cacodylic acid. As before, 5 separate 10 minute washes were performed. The samples were then progressively dehydrated using increasing concentrations of ethanol. Samples were incubated for 20 minutes at each of the following concentrations of ethanol 20%, 30%, 50%, and 70%. Samples were then air dried. Individual pegs were removed using sterile pliers and mounted to SEM pins. Samples were then coated and visualized.

References

Anwar, H., Strap, J.L. and Costerton, J.W. (1992) Susceptibility of biofilm cells of *Pseudomonas aeruginosa* to bactericidal actions of whole blood and serum. FEMS Microbiol. Let. 71: 235-241.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (ed.). (1991) In Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Barta, T.M., Kinscherf, T.G., and Willis, D.K. (1992) Regulation of tabtoxin production by the *lemA* gene in *Pseudomonas syringae*. J. Bacteriol. 174: 3021-3029.

Blummer, C., Heeb, S., Pessi, G., and Haas, D. (1999) Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. Proc. Natl. Acad. Sci. USA. 96: 14073-14078.

Boyd, A., and Chakrabarty, A.M. (1995) *Pseudomonas aeruginosa* biofilms: role of alginate exopolysaccharide. J. Ind. Microbiol. 15:162-168.

Bradley, D.E. (1980) A function of *Pseudomonas aeruginosa* PAO pili: twitching motility. Can. J. Microbiol. 26: 146-154.

Castaneda, M., Guzman, J., Moreno, S., and Espin, G. (2000) The GacS sensor kinase regulates alginate and poly-beta-hydroxybutyrate production in *Azotobacter vinelandii*. J. Bacteriol. 182: 2624-2628.

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D. and Buret, A. (1999) The Calgary Biofilm Device: New Technology for the rapid determination of antibiotic susceptibilities of bacterial biofilms. J. Clin. Microbiol. 37: 1771-1776.

Chancey, S.T., Wood, D.W. and Pierson, L.S. (1999) Two-component transcriptional regulation of N-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. Appl. Environ. Microbiol. 65: 2294-2299.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. (1995) Microbial biofilms. Annu. Rev. Microbiol. 49:711-745.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. (1999) Bacterial biofilms: a common cause of persistent infections. Science. 284: 1318-1322.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W. and Greenberg, E.P. (1998) The involvement of cell-cell signals in the development of a bacterial biofilm. Science. 280: 295-298.

Frank, D.W. and Iglewski, B.H. (1988) Kinetics of *toxA* and *regA* mRNA accumulation in *Pseudomonas aeruginosa*. J. Bacteriol. 170: 4477-4483.

Gambello, M.J., and IglewskI, B.H. (1991) Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J. Bacteriol. 173: 3000-3009.

Grewal, S.I.S., Han, B. and Johnstone, K. (1995) Identification and characterization of a locus which regulates multiple functions in *Pseudomonas tolaasii*. the cause of brown blotch disease of *Agaricus bisporus*. J. Bacteriol. 177: 4658-4668.

Hirano, S.S., Charkowski, A.O., Collmer, A., Willis, D.K. and Upper, C.D. (1999) Role of Hrp type III secretion system in growth of *Pseudomonas syringae* pv. syringae B728a on host plants in the field. Proc. Natl. Acad. Sci. USA. 96: 9851-9856.

Holloway, B.W., Krishnapillai, V. and Morgan, A.F. (1979) Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. 43: 73-102.

Kohler, T., Curty, L.K., Barja, F., Van Delden, C. and Pechere, J.C..(2000) Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J Bacteriol. 182:5990-5996.

Jander, G., Rahme L.G. and Ausubel, F.M. (2000) Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J. Bacteriol. 182: 3843-3845.

Jensen, E.T., Kharazami, A., Lam, K., and Costerton, J.W., Hoiby, N. (1990) Human polymorphonuclear leukocyte response to *Pseudomonas aeruginosa* grown in biofilms. Infect. Immun. 58: 2383-2385.

Jensen, E.T., Kharazami, A., Hoiby, N. and Costerton, J.W. (1992) Some bacterial parameters influencing the neutrophil oxidative burst response to *Pseudomonas aeruginosa* biofilms. APMIS. 100: 727-733.

Jensen, E.T., Kharazmi, A., Garred, P., Kronberg, G., Fomsgaard, A., Mollnes T.E. and Hoiby, N. (1993) Complement activation by *Pseudomonas aeruginosa* biofilms. Microbial Path. 15: 377-388.

Johnston, C., Pegues, D.A., Hueck, C.J., Lee, A. and Miller, S.I. (1996) Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. Mol. Microbiol. 22: 715-27.

Kinscherf, T.G. and Willis, D.K. (1999) Swarming by *Pseudomonas syringae* B728a requires *gacS* (*lemA*) and *gacA* but not the acyl homoserine lactone biosynthetic gene *ahlI*. J. Bacteriol. 181: 4133-4136.

Kitten, T., Kinscherf, T.G., McEvoy, J.L. and Willis, D.K. (1998) A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. Mol. Microbiol. 28: 917-929.

Lam, J.S., Chan, R., Lam, K. and Costerton, J.W. (1980) Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infect. Immun. 28: 546-556.

Liao, C.H., McCallus, D.E. and Fett, W.F. (1994) Molecular characterization of two gene loci required for production of the key pathogenicity factor for pectate lyase in *Pseudomoans viridiflava*. Mol. Plant – Microbe Interact. 7: 391-400.

Liao, C.H., McCallus, D.E., Wells, J.M., Tzean, S.S., and Kang, G.Y. (1996) The *repB* gene required for production of extracellular enzymes and fluorescent siderophores in *Pseudomonas viridiflava* is an analog of the *gacA* gene in *Pseudomonas syringae*. Can. J. Microbiol. 42: 177-182.

Liss, L. (1987) New M13 host: DH5αF' competent cells. Focus 9: 13.

Mahajan-Miklos, S., Tan, M.W., Rahme, L.G. and Ausubel, F.M. (1999) Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa* - *Caenorhabditis elegans* pathogenesis model. Cell. 96: 47-56.

May, T.B., Chakrabarty, A.M. (1994) Isolation and assay of *Pseudomonas aeruginosa* alginate. Methods in Enzymology. 235:295-304

Mcluicni, G.J., Grout, M., Evans, D.J. and Pier, G.B. (1995) Mucoid *Pseudomonas aeruginosa* growing in a biofilm *in vitro* are killed by opsonic antibodies to the mucoid exopolysaccharide capsule but not antibodies produced during chronic lung infection in cystic fibrosis patients. J. Immunol. 155: 2029-2038.

Miller, J.H. (1972) In Experiments in Molecular Genetics. Cold Spring Harbour, Cold Spring Harbour Laboratory Press.

Nichols, W.W., Evans, M.J., Slack, M.P.E. and Walmsley, H.L. (1989) The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*. J. Gen. Microbiol. 135: 1291-1303.

O'Toole, G.A., and Kolter, R. (1998a) Initiation of biofilm formation in *Pseudomonas flourescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. 28: 449-461.

O'Toole, G.A., and Kolter, R. (1998b) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. 30: 295-304.

O'Toole, G.A., Gibbs, K.A., Hager, P.W., Phibbs, P.V. and Kolter, R. (2000) The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. J. Bacteriol. 182: 425-431.

Pearson, J.P., Gray, K.M., Passador, L., Tucker, K.D., Eberhard, A., Iglewski, B.H. and Greenberg, E.P. (1994) Structure of the autoinducer required for the expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. USA 91: 197-201.

Pearson, J.P., Pesci, E.C. and Iglewski, B.H. (1997) Roles of *Pseudomonas aeruginosa las* and *rhl* quorum sensing systems in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. 179: 5756-5767.

Pesci, E.C., Pearson, J.P., Seed, P.C. and Iglewski, B.H. (1997) Regulation of *las* and *rhl* quorum-sensing in *Pseudomonas aeruginosa*. J. Bacteriol. 179: 3127-3132.

Pratt, L.A. and Kolter, R. (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. 30: 285-293.

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G. and Ausubel, F.M. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. Science. 268: 1899-1902.

Rahme, L.G., Tan, M.W., Le, L., Wong, S.M., Tompkins, R.G., Calderwood, S.B. and Ausubel, F.M. (1997) Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors. Proc. Natl. Acad. Sci. USA. 94: 13245-13250.

Rahme, L.G., Ausubel, F.M., Cao, H., Drenkard, E., Goumnerov, B.C., Lau, G.W., Mahajan-Miklos, S., Plotnikova, J., Tan, M.W., Tsongalis, J., Walendziewicz, C.L. and Tompkins, R.G. (2000) Plants and animals share functionally common bacterial virulence factors. Proc. Natl. Acad. Sci. USA. 97: 8815-8821.

Rashid, M.H., Rumbaugh, K., Passador, L., Davies, D.G., Hamood, A.N., Iglewski, B.H. and Kornberg, A. (2000) Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA. 97: 9636-9641.

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A. and Haas, D. (1997) The global activator GacA of *Pseudomonas aeruginosa* PAO

positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide and lipase. Mol. Microbiol. 24: 309-319.

Rich, J.J., Kinscherf, T.G., Kitten, T. and Willis, D.K. (1994) Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. J. Bacteriol. 176: 7468-7475.

Rumbaugh, K.P., Griswold, J.A. and Hamood, A.N. (1999a) Contribution of the regulatory gene *lasR* to the pathogenesis of *Pseudomonas aeruginosa* infection of burned mice. J. Burn Care Rehabil. 20: 42-49.

Rumbaugh, K.P., Griswold, J.A., Iglewski, B.H. and Hamood, A.N. (1999b) Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. Infect. Immun. 67: 5854-5862.

Seed, P.C., Passador, L. and Iglewski, B.H. (1995) Activation of the *Pseudomonas aeruginosa lasI* gene by LasR and the Pseudomonas autoinducer PAI: and Autoinduction regulatory hierarchy. J. Bacteriol. 177: 654-659.

Semmler, A.B., Whitchurch, C.B. and Mattick, J.S. (1999) A re-examination of twitching motility in *Pseudomonas aeruginosa*. Microbiol. 145: 2863-2873.

Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J. and Greenberg, E.P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407:762-764.

Smith, A.W. and Iglewski, B.H. (1989) Transformation of *Pseudomonas aeruginosa* by electroporation. Nucl. Acids Res. 17: 10509.

Stewart, P.S. (1994) Biofilm accumulation model that predicts antibiotic resistance of *Pseudomonas aeruginosa* biofilms. Antimicrob. Agents Chemother. 38: 1052-1058.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey,M.J., Brinkman, F.S., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K., Wu, Z. and Paulsen, I.T. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature. 406:959-964.

Suci, P.A., Mittelman, M.W., Yu, F.P. and Geesey, G.G. (1994) Investigation of ciprofloxacin penetration into *Pseudomonas aeruginosa* biofilms. Antimicorb. Agents Chemother. 35: 2125-2132.

Schweizer, H.P. (1991) *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene 97:109-112.

Tan, M.W., Rahme, L.G., Sternberg, J.A., Tompkins, R.G. and Ausubel, F.M. (1999a) *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. Proc. Natl. Acad. Sci. USA. 96: 2408-2413.

Tan, M.W., Mahajan-Miklos, S. and Ausubel, F.M. (1999b) Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. Proc. Natl. Acad. Sci. USA. 96: 715-720.

Tang, H.B., DiMango, E., Bryan, R., Gambello, M., Iglewski, B.H., Goldberg, J.B. and Prince, A. (1996) Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. Infect. Immun. 64: 37-43.

Whistler, C.A., Corbell, N.A., Sarniguet, A., Ream, W. and Loper, J.E. (1998) The two component regulators GacS and GacA influence accumulation of stationary phase sigma factor σ^S and the stress response in *Pseudomonas fluorescens* Pf-5. J. Bacteriol. 180: 6635-6641.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119.

Zhang, J.P. and Normark, S. (1996) Induction of gene expression in *Escherichia coli* after pilus mediated adherence. Science. 273: 1234-1236.

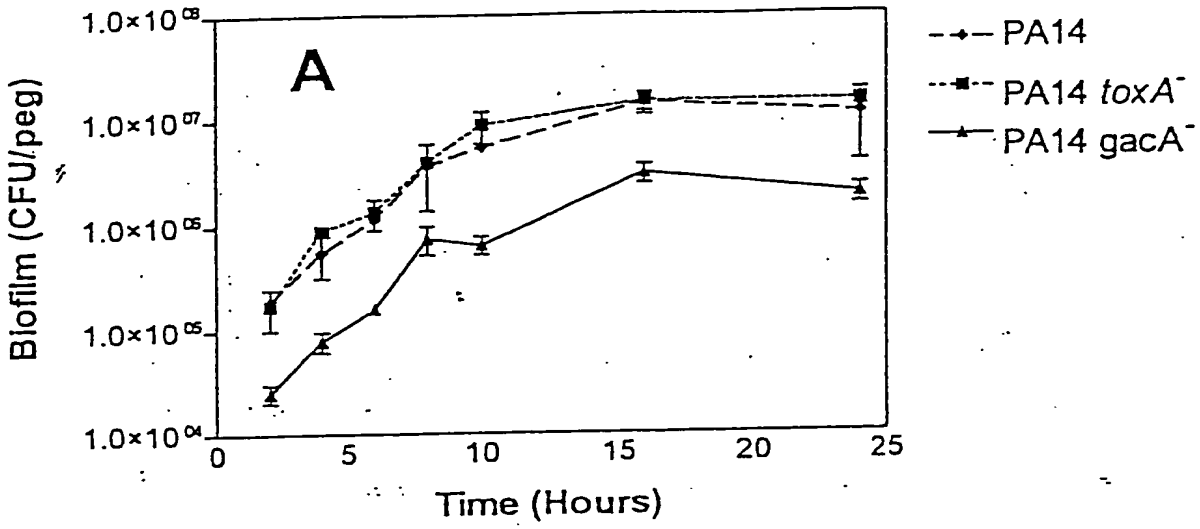
Fig. 1A. Biofilm formation rates. Biofilm growth curves were performed on *P. aeruginosa* strains PA14 wild type (◆), PA14 *toxA*⁻ (■) and PA14 *gacA*⁻ (▲) in the MBEC™ device over a 24 hour period.

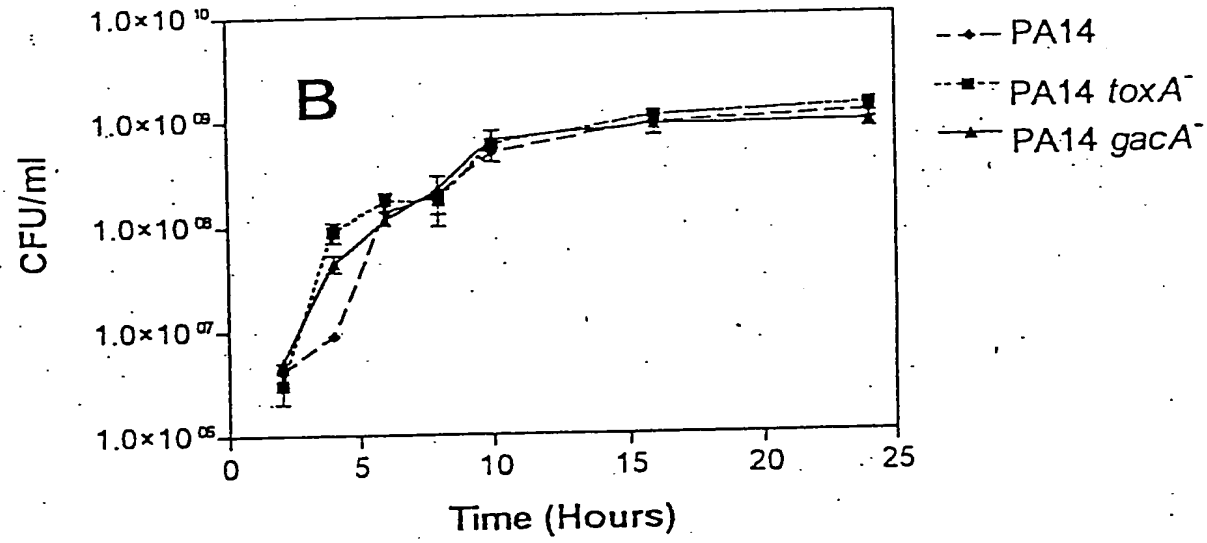
Fig. 1B. Planktonic population growth rates. Proliferation rates of the planktonic populations of *P. aeruginosa* strains PA14 wild type (◆), PA14 *toxA*⁻ (■) and PA14 *gacA*⁻ (▲) in the MBEC™ device over a 24 hour period.

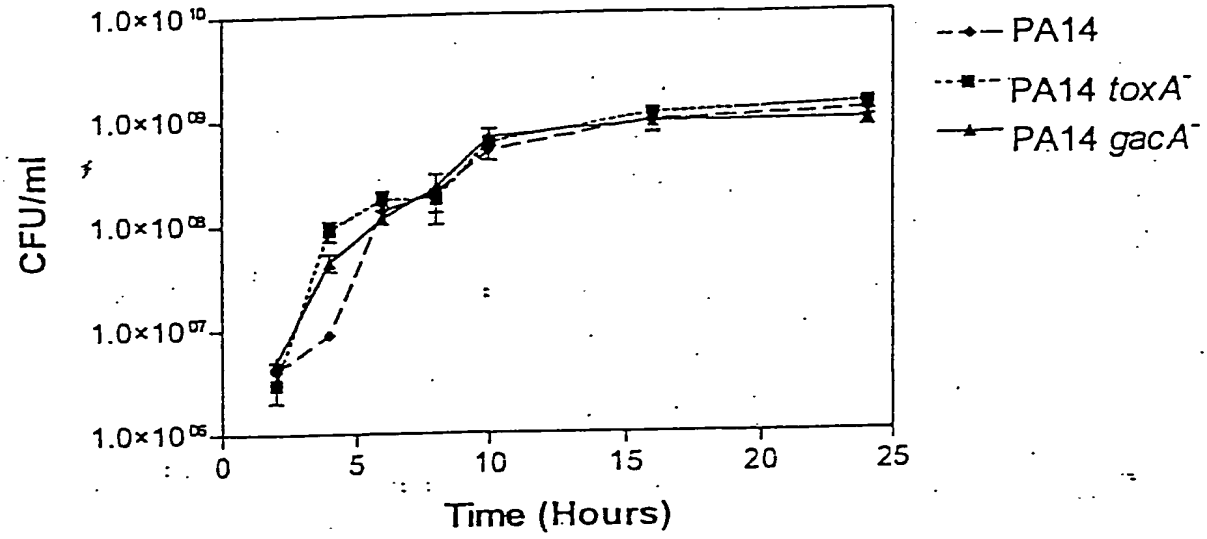
Fig. 2. Biofilm growth rates of PA14 strains over-expressing *gacA*. The rate of biofilm growth of *P. aeruginosa* strains PA14 wild type (pUCP-*gacA*)(◆), PA14 *toxA*⁻ (pUCP-*gacA*) (■) and PA14 *gacA*⁻ (pUCP-*gacA*) (▲) were monitored for a 24 hour period in the MBEC™ device.

Fig.3. High and low magnification scanning electron micrographs of biofilms formed by PA14 wild type (A-B), PA14 *toxA*⁻ (C-D) and PA14 *gacA*⁻ (E-F) after 24 hours of growth. PA14 *gacA*⁻ adheres to the peg, however, fails to aggregate to form microcolonies and develop mature biofilm structures. (A,C,E=high magnification), (B,D,F=low magnification).

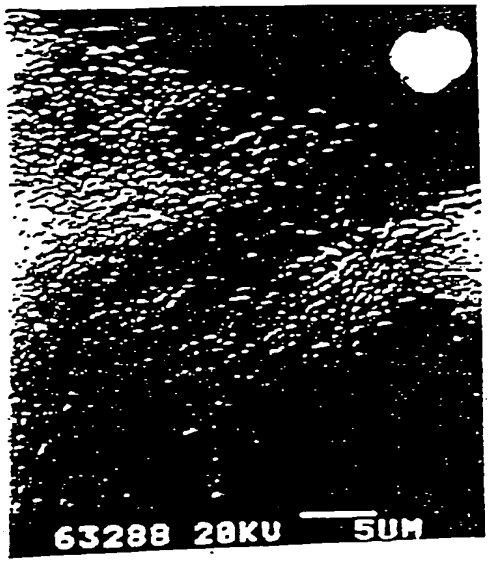
Fig.4 Biofilm antibiotic resistance. Biofilms of *P. aeruginosa* PA14 *toxA*⁻ and PA14 *gacA*⁻ were challenged for 16-20 hours with various concentrations of 5 antibiotics and then sonicated to release individual cells. Surviving biofilm size was determined following serial dilution, and the concentration of antibiotic required to produce a 1000 fold reduction in biofilm mass (relative to control biofilms) is plotted.


A
Biofilm (CFU/peg)
1.0×10 08
1.0×10 07
1.0×10 06
1.0×10 05
1.0×10 04
0
5
10
15
20
25
Time (Hours)
PA14
PA14 toxA-
PA14 gacA-


B
CFU/ml
1.0×10 10
1.0×10 09
1.0×10 08
1.0×10 07
1.0×10 06
0
5
10
15
20
25
Time (Hours)
PA14
PA14 toxA-
PA14 gacA-


1.0×10 10
1.0×10 09
1.0×10 08
1.0×10 07
1.0×10 06
CFU/ml
0
5
10
15
20
25
Time (Hours)
PA14
PA14 toxA-
PA14 gacA-

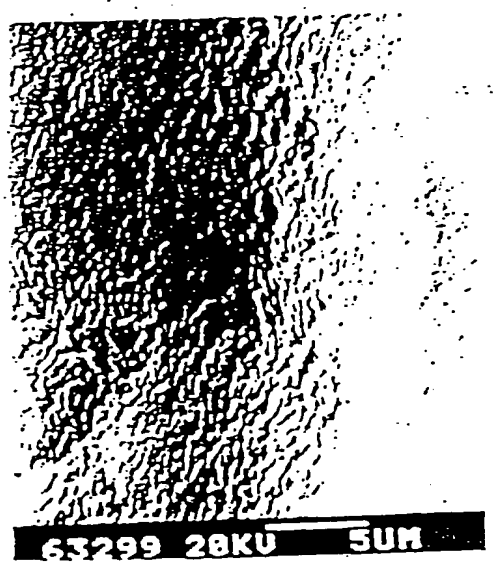
A


63288 20KV 5UM

B


63289 20KV 50UM

C


63299 20KV 5UM

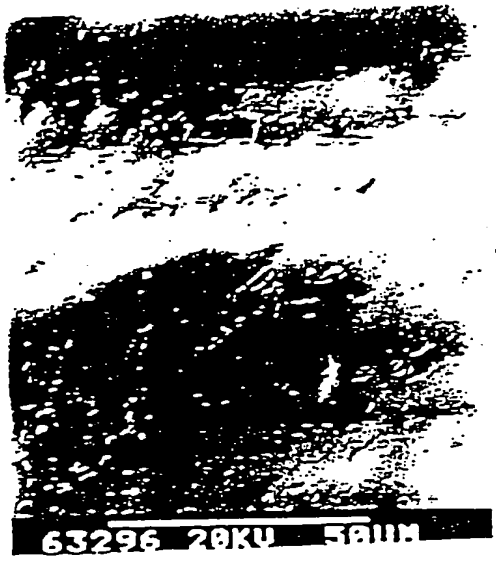
D


63301 20KV 50UM

E


63295 20KV 5UM

F


63296 20KV 50UM

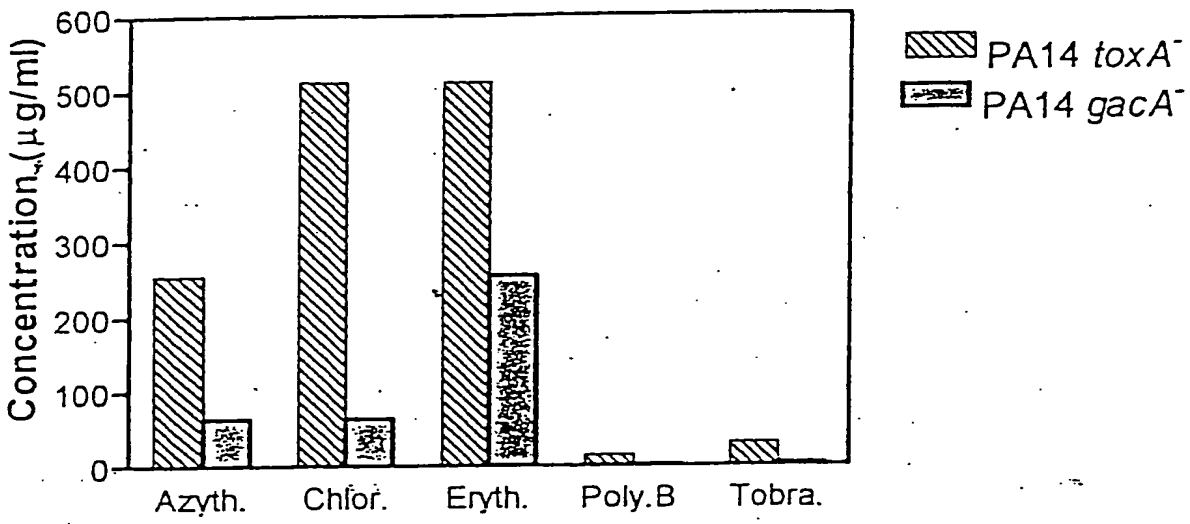

600
500
400
300
200
100
0
Concentration (µg/ml)
Azyth.
Chlor.
Eryth.
Poly.B
Tobra.
PA14 toxA⁻
PA14 gacA⁻

Table 1: Physiological properties of each PA14 derivative

Category	PA14 Wild Type	PA14 *toxA*⁻	PA14 *gacA*⁻
Motility (mm)			
Twitching (24 hr)	8.5 ± 1.2	10.5 ± 0.7	9.3 ± 1.0
Twitching (48 hr)	17.3 ± 1.3	18.7 ± 1.5	17.7 ± 1.5
Swarming (72hr)	22 ± 7.5	16 ± 11	58 ± 11
Flagellar Swimming (72hr)	46 ± 8.6	46 ± 3.2	47 ± 6.3
Autoinducer production (Miller units)			
3-oxo-C12-HSL	10568 ± 29	9091 ± 37	8852 ± 25
C4-HSL	165.5 ± 6.17	158.5 ± 3.8	149.4 ± 3.3
Alginate Production (mg alginate/mg protein)	0.614 ± 0.34	N/A	0.771 ± 0.54

Table 2: Antibiotic susceptibility profile of *P. aeruginosa* PA14 Wild Type, PA14 *toxA*⁻ and PA14 *gacA*⁻.

Antibiotic	PA14 WT		PA14 *toxA*⁻		PA14 *gacA*⁻	
	MIC	MBEC	MIC	MBEC	MIC	MBEC
Azythromycin	64	≥ 1024	64	≥ 1024	64	512
Chloramphenicol	64	≥ 1024	64	≥ 1024	32	1024
Ciprofloxacin	≤ 2	2	≤ 2	2	≤ 2	≤ 2
Erythromycin	128	≥ 1024	128	≥ 1024	64	512
Piperacillin	16	≥ 1024	64	≥ 1024	16	256
Tetracycline	16	512	16	512	16	256
Tobramycin	≤ 2	16	≤ 2	16	≤ 2	16

Table 3: Bacterial strains and plasmids used in this work

Strain/Plasmid	Relevant Characteristics	Reference
Strain		
E. coli		
DH5α	φ80 Δ*lacZ* ΔM15 Δ(*lacZYA-argF*) U169 *recA*1 *endA*1 *hsdR*17 ($r_K^- m_K^+$) *supE*44 *thi*⁻ *d*⁻	Liss, 1987
JM109	*endA*1 *recA*1 *gyrA*96 *thi*, *hsdR*17 ($r_K^- m_K^-$) *relA*1 *supE*44 Δ(*lac-proAB*) [F', *traD*36, *proAB*, *lacI*^{q}*Z* ΔM15]	Yanisch-Perron *et al.*, 1985
MG4	*Δ(argF-lac) U169 zah-735::Tn10 recA56 srl::Tn λ::lasIp-lacZ*	Seed *et al.*, 1995
P. aeruginosa		
UCB-PP PA14	human isolate; able to elicit severe disease in plant and animal models	Rahme *et al.*, 1995
PA14 *toxA*⁻	PA14 Δ*toxA*::Gm^R	Rahme *et al.*, 1995
PA14 *gacA*⁻	PA14 Δ*gacA*::Gm^R	Rahme *et al.*, 1995
PAO1	prototrophic lab strain	Holloway *et al.*, 1979
PAO-JP2	PAO1 Δ*lasI*::Tet, Δ*rhlI*::Tn501-2	Pearson *et al.*, 1997
Plasmids		
pMJG1.7	PSW200 with 1.7kb *Scl*I-*Eco*R1 fragment from pMG3.9 in pUC181.8; contains *lasR*; Ap^R	Gambello and Iglewski, 1991
pUCP18	cloning vector, contains stabilizing fragment for *P. aeruginosa* replication; Ap^R	Schweizer, 1991
pUCP-*gacA*	pUCP18 containing PCR amplified *gacA* from PAO1 genome; Ap^R	

pUC181.8	cloning vector, contains stabilizing fragment for *P. aeruginosa* replication; Ap^R	Frank and Iglewski, 1988
pECP61.5	pJPP8 containing the *rhlA'-lacZ* fusion from pECP60, p^{tac}-*rhlR*; Ap^R	Pesci *et al.*, 1997
pKDT17	pTS400 with p^{lac}-*lasR*; Ap^R	Pearson *et al.*, 1997